

Controlling the Size and Film Strength of Individualized Cellulose Nanofibrils Prepared by Combined Enzymatic Pretreatment and High Pressure Microfluidization

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The production of functionalized polymers from biomass is of great interest. Cellulose nanofibrils (CNFs) isolated from lignocellulose have great potential in novel functional materials. In the present study, mild enzymatic treatment followed by high pressure microfluidization of a bleached softwood kraft pulp led to the release of individualized CNFs. Disk milling and high pressure microfluidization resulted in entangled networks of CNFs. CNFs from mild enzyme pretreatments were 8 to 12 nm in diameter and 200 to 400 nm in length, while CNFs from pure mechanical pretreatment were an entangled network of nanofibrils with a diameter of 10 to 20 nm. Films prepared from the resulting CNFs were flexible and semitransparent, and they exhibited high specific tensile stress and modulus. The specific tensile stress and modulus were increased by 3- to 5-fold and 5- to 11-fold, respectively. The specific tensile modulus of the CNFs films from mild enzyme treatments followed by microfluidization was approximately 15 to 16 MN·m/kg, while that of CNFs from pure mechanical fibrillation with or without microfluidization was 10 MN·m/kg and 14 MN·m/kg, respectively. The specific tensile strength of the CNFs films from mild enzyme treatment was slightly lower (72 to 98 kN·m/kg) than that of the CNFs films from pure mechanical fibrillation.

Keywords: Mechanical fibrillation; Microfluidization; Grinding; Enzymatic treatment; Cellulose nanofibrils; Mechanical strength

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INTRODUCTION

Cellulose nanofibrils (CNFs) are suitable raw materials for novel functional materials because of their excellent physical and mechanical properties (Klemm *et al.* 2011; Moon *et al.* 2011; Wang *et al.* 2013). Functional materials including fiber, films, membranes, aerogels, scaffolds, and hybrid composites have been developed from CNFs (Wang *et al.* 2014; Tang *et al.* 2015). CNFs are usually isolated by mechanical fibrillation using a homogenizer (Pääkkö *et al.* 2007; Spence *et al.* 2011), microfluidizer (Wang *et al.* 2013), or ultra-fine friction grinder (Wang *et al.* 2012a). The liberation of CNFs by physical pretreatment of untreated cellulosic materials requires a lot of energy, *i.e.*, 10 to 40 kWh/kg (Spence, *et al.* 2011; Wang and Zhu 2015). A wide variety of pretreatment techniques have been used to lower energy consumption. Chemical pretreatments using acidic or alkaline

conditions disintegrate the dense structure of cellulose, but they also cut the cellulose chain randomly, which has a negative effect on the length of the isolated cellulose nanofibrils.

Enzymes that target specific substrates, such as cellulase, hemicellulase, and laccase, are used to modify cellulosic fiber for various applications in the pulp and paper industry (Lee *et al.* 2007; Gehmayr and Sixta 2012). Enzymatic pretreatment of cellulosic material before mechanical shearing reduces the energy required for CNF isolation. Monocomponent cellulase or complex cellulase selectively hydrolyzes certain cellulosic structures (Verma *et al.* 2015). Residual cellulose that is recalcitrant to enzymatic pretreatment is more easily isolated into nanoscale fibers (Zhu *et al.* 2011). Morphological properties and the aspect ratio of nanocellulose produced from recalcitrant cellulose could be enhanced compared with CNFs produced by acid hydrolysis. Cellulase used for pretreatment during the production of CNFs is easily recycled by filtration, desorption, and re-adsorption (Wang *et al.* 2012b). The production of CNFs by combined mild enzymatic hydrolysis and mechanical fibrillation has been recently reported (Henriksson *et al.* 2007; Pääkkö *et al.* 2007; Siddiqui *et al.* 2011). There are different sources of cellulase enzymes including commercial or purified endoglucanase, complex cellulase cocktails, or fungi (Janardhnan and Sain 2007; Janardhnan and Sain 2011; Nechyporchuk *et al.* 2014; Wang *et al.* 2014; Teixeira *et al.* 2015). However, previous studies lack detailed investigations of the strength properties of films generated from CNFs isolated *via* mild enzymatic hydrolysis.

Previously, individualized CNFs were produced from residual cellulose recovered from the waste stream of acid hydrolysis (Wang *et al.* 2013). This acid treatment was hydrolytic and decreased the fiber length and degree of polymerization. In the present work, a relatively greener enzymatic pretreatment step was applied to isolated CNFs using a never-dried, bleached softwood kraft pulp. The effects of complex cellulase and endoglucanase loading on the properties of cellulose nanofibrils were investigated. The morphology of CNFs before and after microfluidization was compared by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The degree of polymerization (DP) and the crystallinity index (CrI) were also determined as indicators of CNF quality. CNF films were fabricated by ultrafiltration of CNF suspensions. Optical and mechanical properties of the films were also characterized to elucidate the effects of pretreatments on final CNF film properties.

EXPERIMENTAL

Pulp and Enzymes

Never-dried, bleached softwood kraft pulp (BSKP) was obtained from the Institute of Paper Science and Technology, Georgia Institute of Technology, Atlanta, USA. Its chemical components included glucan ($82.2 \pm 2.5\%$), xylan ($8.4 \pm 0.2\%$), and mannan ($6.84 \pm 1.8\%$). The average fiber length was 2.19 ± 0.06 mm. Celluclast 1.5 L (a complex cellulase mixture of endoglucanase, exoglucanase, and cellobiase) and Fibercare (extraglucanase-deficient endoglucanase) were obtained from Novozymes North America Inc. (Franklinton, NC, USA) and were used as received without further purification. All other chemicals were of analytical grade.

Enzymatic Pretreatment

Enzymatic pretreatments were performed in a 250-mL flask in a shaker at 10% (w/v) solid loading at 50 °C, for example, 10 g of pulp in 100 mL of pH 4.8 buffer for 48 h. The enzyme loading is listed in Table 1. Pretreated fibers were collected by ultrafiltration using a membrane with 0.45- μ m pores.

Table 1. Processing Parameters for Enzymatic Pretreatments of BSKP Fiber

Sample ID	E-0	E-1	E-2	E-3	E-4	E-5
Celluclast 1.5L (FPU/g pulp OD)	0	3	3	3	2	1
Fibercare (IU/g pulp OD)	3	0	3	5	1	2
Pretreatment yield (%)	97.8 $\pm$ 0.5	63.5 $\pm$ 0.1	66.8 $\pm$ 0.4	69.2 $\pm$ 0.3	68.7 $\pm$ 0.4	76.6 $\pm$ 0.1

Microfluidization of the Fibers

Homogenization of cellulose fibers after enzymatic pretreatment was performed at a 1.5% solid consistency using a microfluidizer (M-110EH-30 Microfluidics, Newton, MA, USA). Fiber suspensions were passed through the microfluidizer 30 times with a 200 μ m chamber, and 20 additional times with an 87- μ m chamber.

Fiber Length Reduction by Grinder

The BSKP fiber without pretreatment was too long to be directly fed into the microfluidizer. Therefore, the fiber was disk-milled before microfluidization using a Supermasscolloider (model MKZA6-2, Masuko Sangyo Co., Ltd., Kawaguchi, Japan) as previously described (Wang *et al.* 2012a; Wang and Zhu 2015). The gap between the disks was adjusted to -50 μ m from motion zero position, which was determined by slight contact between grinding disks. Pulp slurry was passed through the grinder for up to 2 h (approximately 14 times) at 1.5% consistency. The ground fiber was further disintegrated by the microfluidizer. The samples after enzymatic pretreatment, grinder milling, and microfluidization were designated as samples E, G, and M, respectively (Table 2).

Preparation of CNFs Films

At least five CNF films were prepared for each sample. CNF gel was diluted to 0.1% consistency and continuously stirred for 12 h. Films were formed by ultrafiltration of CNF slurry using a 142-mm Millipore hazardous waste filtration system with a 0.22- μ m PVDF membrane (Millipore, GVWP14250, Tullagreen, Ireland). CNF film and plot paper were pressed at 30 psi for 3 min and then for another 3 min at 50 psi. Pressed CNF film was dried overnight in an incubator at 60 °C with a 50 pound load on top.

Optical and Mechanical Analyses of CNFs Films

The opacity of the films was calculated according to TAPPI T519 om-06 (2006). Strength tests were conducted using an Instron system (model 5865, Norwood, MA, USA) equipped with a LX500 laser extensometer (MTS System Corporation, Eden Prairie, MN, USA) for precise displacement determination. Prior to mechanical testing, CNF films were pre-conditioned. Rectangular tensile specimens were 15 mm wide with an extensometer gauge length of 40 mm and a nominal grip length of 60 mm.

Determination of Degree of Polymerization (DP) and Crystallinity Index (CrI)

The degree of polymerization ($DP^{0.905}$) was measured using a capillary viscometer according to the TAPPI T230 om-08 standard (2008) and was calculated using Eq. 1 (Mazumder *et al.* 2000),

$$DP^{0.905} = 0.75[954 \log(X) - 325] \quad (1)$$

where X is the viscosity of cellulose.

The crystallinity index was determined by a Bruker RFS 100 Spectrometer (Bruker Instruments Inc., Billerica, MA, USA) and calculated according to Eq. 2 (Agarwal *et al.* 2010),

$$CrI = [(I_{380}/I_{1096}) - 0.0286]/0.0065 \quad (2)$$

where I_{380} and I_{1096} are the band intensities at 380 and 1096 cm^{-1} , respectively.

Microscopy

Specimens for SEM were examined by a Zeiss EVO 40 SEM (Carl Zeiss NTS, Peabody, MA, USA). For TEM, specimens were deposited and dried on sample grids that contained ultrathin carbon films supported by thicker carbon grids (EMS CF200-Cu grid). A Hitachi H7650 microscope (Tokyo, Japan) with an accelerating potential of 80 keV was used.

The fiber diameters and lengths were measured in SEM or TEM images using ImageJ software (<http://rsbweb.nih.gov/ij/>).

RESULTS AND DISCUSSION

Morphology of BSKP Fibers

SEM revealed BSKP fiber morphologies (Fig. 1a). Untreated BSKP fiber could not be disintegrated by the microfluidizer and instead caused severe clogs. Long fibers blocked the inlet reservoir and chamber (200 μm) of the system, which stopped the flow and prevented fibrillation of the suspension. BSKP fiber frequently clogged the tube, and the device had to be emptied and cleaned.

Because it was impossible to homogenize at a consistency of 0.3% or lower, disk milling or mild enzymatic hydrolysis was used to reduce fiber length and to avoid blocking the microfluidizer tube.

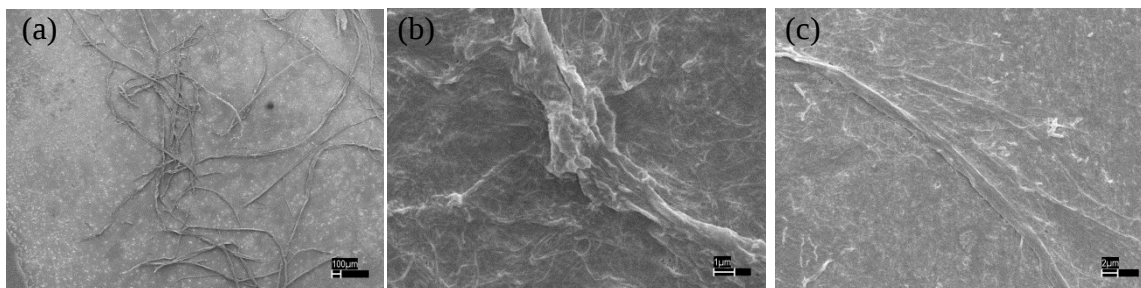


Fig. 1. SEM images of BSKP fiber before and after grinder treatment. (a) Untreated. Scale bar = 100 μm . (b, c) grinder-treated at different magnifications. Scale bar = 1 μm for b and 2 μm for c

Fibrils Morphology after Disk Milling or Enzymatic Pretreatments

A white milky gel was soon formed after several passes through the grinder. Disk milling effectively cut the fibers and broke the fiber cell walls into fragments (Figs. 1b, c). Grinder pre-fibrillation treatment was ended after 2 h, with approximately 3 KWh/Kg energy consumed. Further grinding did not show any noticeable change (Wang *et al.* 2012a); large nanofibrils bundles were still present (Fig. 1c). The nanofibril bundles were 2 μm in diameter and hundreds of μm in length. Disk milling alone was not sufficient to separate cellulose nanofibrils uniformly. These highly entangled structures could not be distinguished under SEM, as they had limited dispersion and resolution. Fibrils produced with disk milling were examined in more detail using TEM.

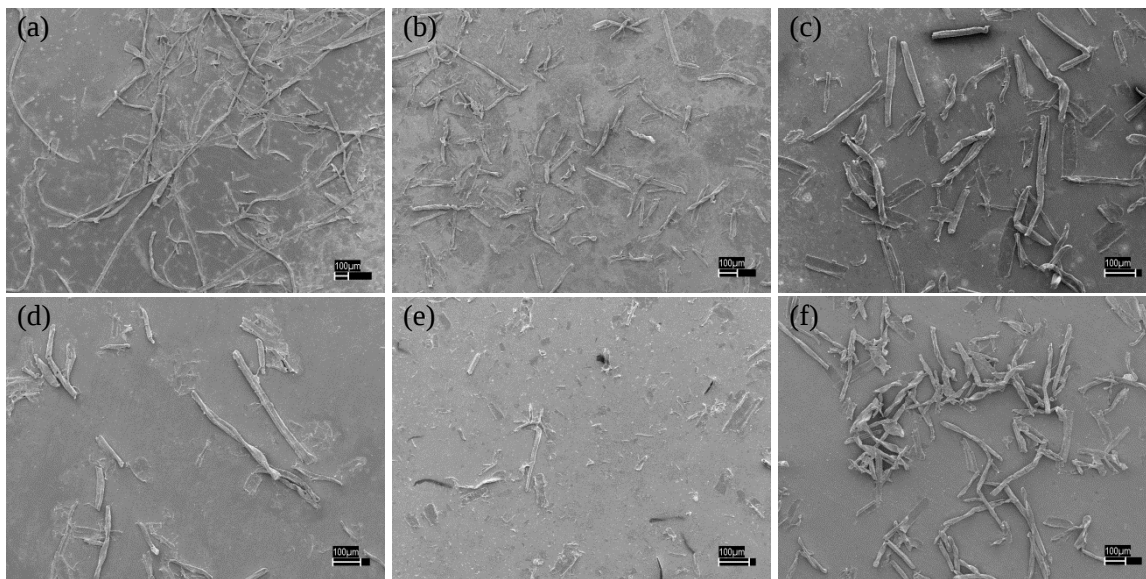


Fig. 2. SEM of BSKP fiber after enzymatic pretreatments with different Celluclast 1.5 L and Fibercare loadings. (a) 0/3; (b) 3/0; (c) 3/3; (d) 3/5; (e) 2/1; (f) 1/2. Scale bar = 100 μm

There were obvious morphological differences between BSKP fibers treated with different blends of Celluclast 1.5 L and Fibercare (Fig. 2). Fibers treated with Fibercare remained almost intact. Limited cell swelling was detected, and only a few fines of 100 to 200 μm in length were observed. Fiber treated with Celluclast 1.5 L alone or combined with Fibercare produced relatively uniform and shorter fibers of approximately 200 to 300 μm . SEM images showed that fibers were cleaved by enzyme treatments (Figs. 2b, c, f); in one case, the fiber cell walls were completely broken into fragments and fines (Fig. 2e). In Fig. 2d, both fiber cutting and cell collapse were observed. Celluclast 1.5 L enzymatic hydrolysis was more drastic because it led to a dramatic reduction in DP (Table 2). Even at the lowest loading of 1 FPU/g, the resultant fibers appeared shorter than those from the Fibercare treatment at 3 IU/g loading. The huge morphological differences between Celluclast 1.5 L- and Fibercare-treated BSKP fiber indicated that the two cellulase enzymes worked differently. Fibercare, an extraglucanase deficient endoglucanase, is more selective, and enacts limited hydrolysis of the amorphous domain of cellulose (Wang *et al.* 2012b), while Celluclast 1.5L, a complex cellulase mixture with endoglucanase and exoglucanase activity, provides extensive hydrolysis of amorphous and crystalline cellulose (Rahikainen *et al.* 2011).

Morphological and Structural Properties of CNFs

The white milky gel produced by grinding at 1.5% consistency was directly fed into the microfluidizer. Disk milling greatly facilitated the disintegration of the fiber. Almost no chamber clog was detected during the homogenization of the fiber at a relatively high consistency, *i.e.*, 1.5%. The CNF gel before and after microfluidization was characterized by TEM (Fig. 3). After disk milling, CNFs were entangled fibril networks (Figs. 3a, b), with diameters ranging from 7 to 45 nm (average diameter of 18.9 ± 8.4 nm). Disk milling combined with microfluidization resulted in slightly finer and more uniform structures than grinder milling alone. The diameter distribution of CNFs-GM ranged from 6 to 23 nm, with an average of 13.6 ± 4.5 nm. However, fibril bundles with incomplete separation remained in the sample (Fig. 3c).

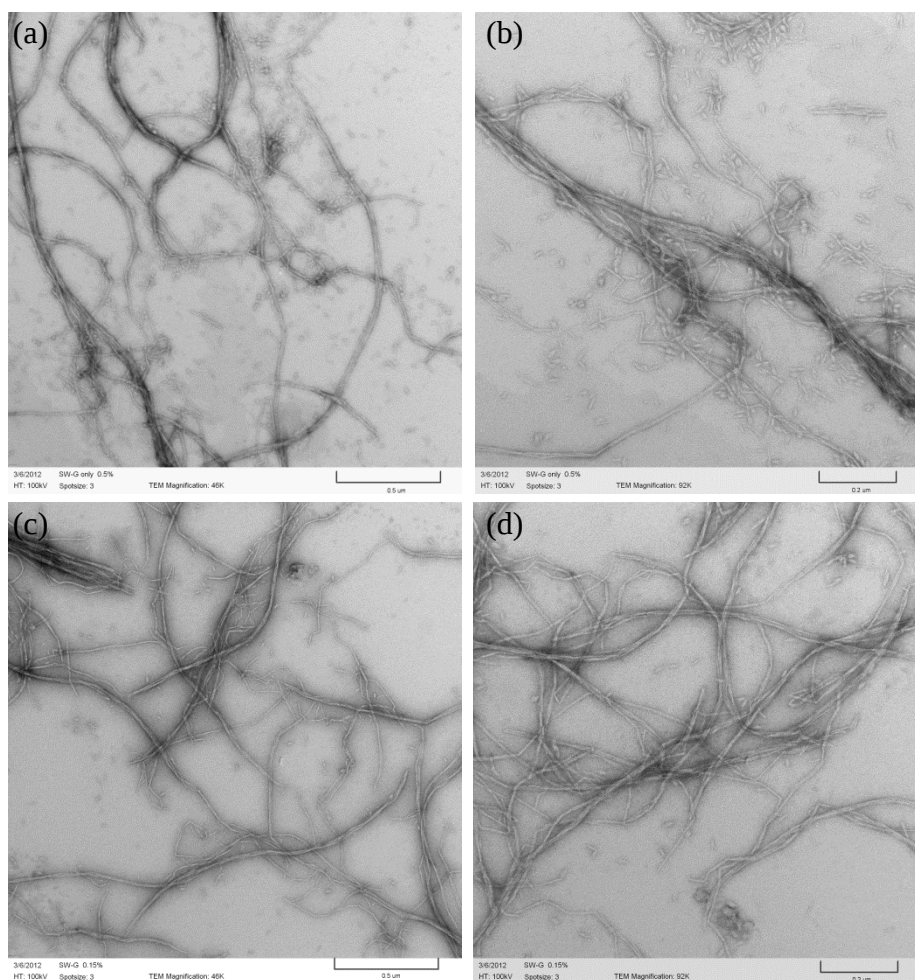


Fig. 3. TEM of BSKP fiber after disk milling and microfluidization. (a) CNFs-G, scale bar = 500 nm; (b) CNFs-G, scale bar = 200 nm; (c) CNFs-GM, scale bar = 500 nm; and (d) CNFs-GM, scale bar = 200 nm

Enzymatic treatments also liberated CNFs from the pulp. All fibers subjected to mild enzyme pretreatment were successfully homogenized. Individualized CNFs were prepared by combined enzymatic pretreatments and high-pressure microfluidization (Fig. 4). Compared to CNFs produced by disk milling and microfluidization, enzymatic

pretreatment resulted in CNFs with smaller diameters and shorter lengths. There were few morphological differences among the six samples of CNFs separated from enzymatically-pretreated fibers. CNFs that were isolated were individual whiskers with relatively uniform diameters, which ranged from 2 to 12 nm, and lengths in the range of 100 to 800 nm. The CNFs exhibited a high aspect ratio (length/diameter) of approximate 50:400. Essentially, the dimension of the fibrils depends on the enzymatic hydrolysis condition. The CNFs-EM-0 gels consisted of individual fibrils with evenly ranged diameters of approximately 5 to 14 nm and lengths of 170 to 820 nm. The length and diameter of the CNFs-EM-1 sample were 303 ± 116 nm and 10 ± 4 nm, respectively; these values for CNFs-EM-2 were 322 ± 172 nm and 10 ± 5 nm, respectively. Compared with other samples pretreated with Celluclast 1.5 L, CNFs-EM-3 and CNFs-EM-5 had relatively longer fibril lengths of 320 ± 170 and 330 ± 130 nm, respectively. CNFs-EM-4 and CNFs-EM-5 exhibited loose aggregate networks.

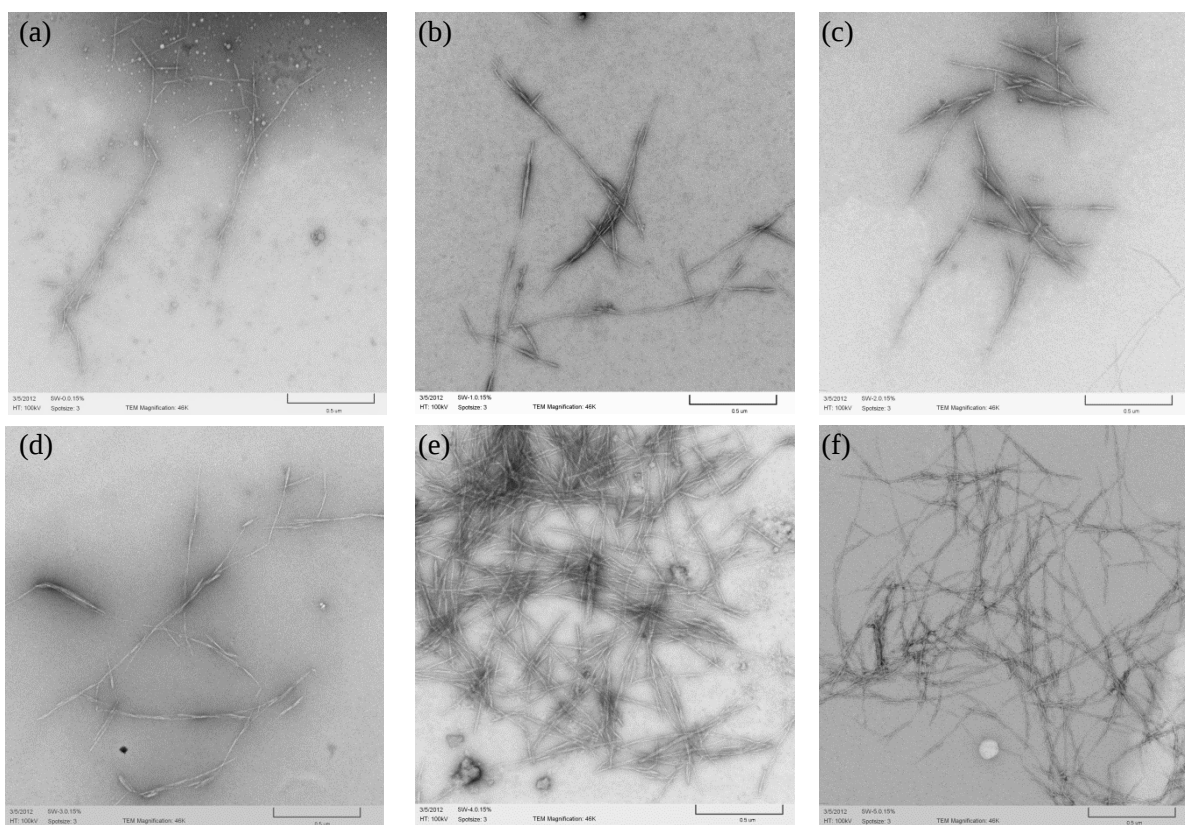


Fig. 4. TEM of CNFs from microfluidization of BSKP fibers with enzymatic treatment. (a) CNFs-EM-0-0/3; (b) CNFs-EM-1-3/0; (c) CNFs-EM-2-3/3; (d) CNFs-EM-3-3/5; (e) CNFs-EM-4-2/1; (f) CNFs-EM-5-1/2. Scale bar = 500 nm

Degree of Polymerization (DP) and Crystallinity Index (CrI)

The DP and CrI of original BSKP fiber and the fibers after enzymatic treatment at different cellulase loadings and mechanical fibrillation are listed in Table 2. Disk milling physically disrupted the ordered cellulose structure, which resulted in lower cellulose crystallinity with short cellulose chain length. The DP of the BSKP fiber decreased rapidly from approximately 1500 to 900 after disk milling. A dramatic decrease in crystallinity from 51.3 ± 2.2 to 39.5 ± 2.7 (a reduction of 23%) was observed after 2 h of milling.

Enzyme mixture treatments also significantly reduced the cellulose DP by 300 to 500, while the CrI increased from 51% to between 54 and 60%, depended on the pretreatment conditions. This result occurred because amorphous cellulose is more susceptible to cellulase enzymes, which subsequently increases the CrI.

The effects of microfluidization on DP and CrI were quite similar to that of disk milling. After microfluidization, the DP decreased to 516 for disk-milled fiber and to approximately 300 for enzyme treated fibers. CrI was reduced to approximately 35% regardless of the enzymatic pretreatment condition. Interestingly, the average length (nm) and DP of CNFs-EM were all approximately 300. The average CNF length is linearly correlated with the DP (Shinoda *et al.* 2012).

Optical and Mechanical Properties of CNFs Films

At least five CNF films for each condition were prepared to test the optical and mechanical properties. Figure 5 presents the opacity values for CNF films. The decrease in fibril dimensions enhanced light transmittance, reducing film opacity. Compared with the original BSKP sheets, enzymatic hydrolysis and mechanical disintegration dramatically reduced the opacity of CNF films. The opacity of films made after disk milling were higher (~30%) than the films made after mild enzyme treatments (24-29%), with the exception of CNFs-EM-1 film (33%). There was no noticeable difference in the opacity values of films made from CNFs pretreated with different enzyme mixtures. A plausible explanation for this observation is that excessive homogenization made the CNF dimensions similar, leading to similar opacity values in the CNF films.

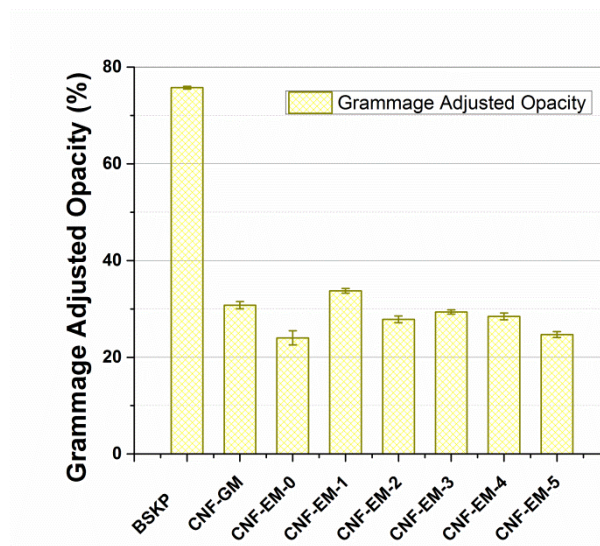
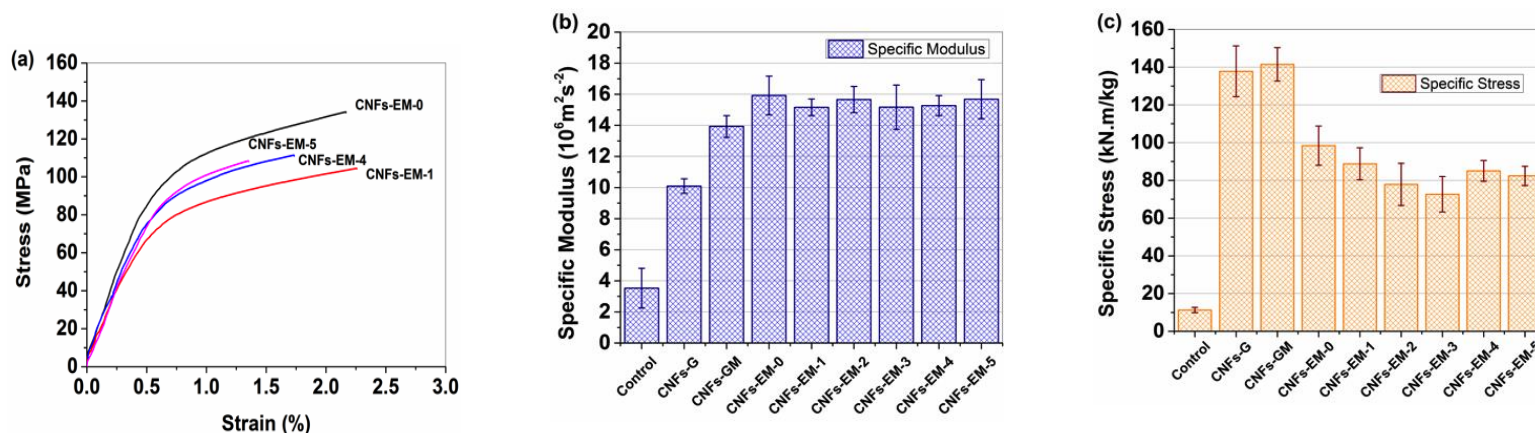


Fig. 5. Comparison of CNF film opacity

Representative stress-strain curves and the average values for specific modulus and tensile strength are shown in Fig. 6. As expected, control films prepared with fiber after enzyme treatment showed low specific tensile strength (11.3 ± 1.4 kN.m/kg) and specific modulus (10.1 ± 0.5 MN.m/kg). Both the specific tensile strength and modulus were greatly enhanced with microfluidization for both enzyme-treated and disk-milled fibers.

Table 2. DP and Crl of Pretreated Fibers and CNFs

Sample ID	DP			Crl		
	Untreated	After Disk Milling/ Enzyme Pretreatment	After Microfluidization	Untreated	After Disk Milling/ Enzyme Pretreatment	After Microfluidization
CNFs-GM	1489.9 ± 0.5	901.4 ± 2.7	516.3	51.3 ± 2.2	39.5 ± 2.7	34.8 ± 0.1
CNFs-EM-0-0/3		520.2 ± 0.1	283.8		54.2 ± 0.9	34.7 ± 0.1
CNFs-EM-1-3/0		400.1 ± 0.6	308.3		59.9 ± 1.7	36.9 ± 0.1
CNFs-EM-2-3/3		352.8 ± 1.0	252.8		59.8 ± 0.8	35.9 ± 0.2
CNFs-EM-3-3/5	1489.9 ± 0.5	336.3 ± 0.1	251.2	51.3 ± 2.2	59.8 ± 1.5	35.9 ± 0.1
CNFs-EM-4-2/1		409.4 ± 0.1	265.3		56.5 ± 1.2	35.9 ± 0.1
CNFs-EM-5-1/2		418.9 ± 0.5	272.9 ± 3.8		57.2 ± 3.5	32.3 ± 0.1

**Fig. 6.** (a) Stress-strain curves of CNF film at a loading rate of 1 mm/min, (b) specific modulus, and (c) specific stress

The specific modulus of CNFs-GM films was greatly improved from 10 MN.m/kg to 14 MN.m/kg. CNF films obtained after enzymatic pretreatments had greater specific modulus, which was approximately 15 MN.m/kg. The ranking of specific modulus agreed with the ranking of DP, such that a lower DP was reflected in a higher specific modulus. This finding is consistent with previous research (Wang *et al.* 2013). The CNFs-G and CNFs-GM films had relatively high specific tensile strengths of 137.8 ± 13.5 kN.m/kg and 141.5 ± 8.9 kN.m/kg, respectively. CNF films with enzymatic treatment exhibited lower specific tensile strength than CNFs-G and CNFs-GM. This result reflected that CNFs from disk grinding were networked with less damage to DP, while CNFs from enzymatic pretreatment were individualized nanowhiskers, which had fewer hydrogen bonds during film forming.

The differences in CNF morphology exhibited limited influence on the strength properties of CNF film. It is possible that less extensive mechanical homogenization may show the difference on CNFs strength. Future studies should focus on the effects of mechanical fibrillation on the optical and mechanical properties of residual cellulose obtained by enzyme treatment.

CONCLUSIONS

1. Mild enzyme pretreatments combined with microfluidization were used to generate CNFs from BSKP fibers. CNFs made from mild enzyme pretreatment were 8 to 12 nm in diameter and 200 to 400 nm in length, while CNFs from pure mechanical pretreatment were an entangled network of nanofibrils with a diameter of 10 to 20 nm.
2. Mild enzyme pretreatment reduced the required mechanical energy input by at least 3 KWh/Kg. The prepared CNF films were flexible and exhibited high specific tensile stress and modulus.
3. The enzyme blend had dramatic effects on the morphological properties, DP, and CrI of cellulosic residual fibrils. However, these differences had limited influence on CNF film strength properties after extensive mechanical homogenization.

ACKNOWLEDGMENTS

This research was partially completed in the laboratory of Dr. Junyong Zhu (Forest Products Laboratory, USDA). The authors gratefully acknowledge financial support from the National Natural Science Foundation of China (31300493), the Young Scholar Program of Jiangsu University, the Open Funding from the State Key Laboratory of Pulp and Paper Engineering, South China University of Technology (201527) and the State Key Laboratory of Molecular Engineering of Polymers, Fudan University (K2016-19). The authors are also thankful for funding from the China Postdoctoral Science Foundation (2015M581740), Jiangsu Planned Projects for Postdoctoral Research (1501072C), Scientific Innovation Research of College Graduates in Jiangsu Province (KYLX_1086), and PAPD.

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Article submitted: October 30, 2015; Peer review completed: January 14, 2016; Revised version received and accepted: January 19, 2016; Published: January 27, 2016.
DOI: 10.15376/biores.11.1.2536-2547